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In vitro propagation and tissue culture of selected monocots

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IN VITRO PROPAGATION AND TISSUE CULTURE OF SELECTED MONOCOTS

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ABSTRACT

A procedure for the *in vitro* propagation of *Lilium regale*, *Hemerocallis*, and *Hippeastrum* varieties was developed. A reliable method for the procurement of excellent explants for initiating cultures was established for a representative member of each group.

L. regale explants were obtained from within quiescent bulbs. Explants grew well *in vitro*, producing callus tissue and bulblets on a variety of media types, even in the absence of exogenous growth regulators. Explants cultured on IAA and kinetin supplemented media exhibited a greater degree of variation of bulblet production and callus proliferation; however, average bulblet production was increased slightly. 2,4-D medium inhibited organogenesis, in general, but promoted callus proliferation at low concentrations (0.5 and 1.0 mg/l).

Hemerocallis explants were derived from the scape and floral bud tissues. Explants grew slowly *in vitro*; necrosis and contamination was often a problem. Plantlet regeneration occurred from both types of explants when cultured on high kinetin (1.0 mg/l) medium. Subsequent experimentation focused on the morphogenetic potential of scape explants, since shoot initiation occurred freely in response to high kinetin medium. 2,4-D medium (1.0 mg/l) promoted growth as a mixture of abnormal roots interspersed with callus. No plantlet regeneration was obtained from this tissue.

Hippeastrum explants were derived from the inflorescence stem tissue within quiescent bulbs. Explants cultured on medium without growth regulators developed callus and bulblets. The callus tissue, however, was not easily separated from the explants and could not be cultured independently. Bulblet production was not significantly enhanced by supplementing the basal medium with IAA and/or kinetin. 2,4-D medium induced prolific root morphogenesis with an occasional bulblet.

The influence of the auxin-cytokinin ratio upon the organogenic potential of explants, using IAA and kinetin, was investigated. *L. regale* and *Hippeastrum* explants were not influenced by auxin-cytokinin manipulations. However, *Hemerocallis* scape explants responded classically by producing shoots on high kinetin medium and roots on high IAA medium.

A reliable procedure for the establishment of all plantlets in soil was developed.

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INTRODUCTION

The genera Hemerocallis (daylily), Lilium (lily), and Hippeastrum (amaryllis) are members of the subclass Monotyledoneae. Species and varieties within these genera are often highly prized, both aesthetically and monetarily. Unfortunately, natural propagation rates often do not meet the demand for new plants, so artificial propagation methods must be employed. The current methods of propagation involve large expenditures of both time and labor, thereby creating higher prices. Lower prices and increased supply could be accomplished by employing an efficient propagation technique involving tissue cultures. The development of *in vitro* propagation methods has been achieved in asparagus (Hasegawa *et al* 1972), alfalfa (Saunders and Bingham 1972), chrysanthemum (Earle and Langhans 1974), maize (Gresshoff and Doy 1973), and recently daylily (Strode and Oglesby 1976; Heuser and Apps 1976). Recent articles (Murashige 1974; Narayanaswamy 1976) summarize progress made in this field of research and list the plants that have been successfully propagated *in vitro*. It is the objective of this study to further the progress made toward the *in vitro* propagation of the monocots Lilium, Hemerocallis, and Hippeastrum by delineating factors influencing the procurement of suitable explants, relative growth of explants on various media types, the effects of growth regulators and hormones, the production of callus, and the development of plantlets.

Initially, monocots had been difficult to establish as *in vitro* cultures. The first culture of a monocot was reported by Loo (1945), who worked with Asparagus officinalis. Excised stem tips were cultured in a

synthetic medium in the light. Growth occurred as the stem elongated in response to light. However, the cultured stems did not produce a callus tissue, roots, or additional shoots. Such a culture is termed an organ culture rather than a tissue culture. Morel and Wetmore (1951) are credited with the first true tissue culture of a monocot. The tuber of Amorphophallus (Hydrosme) Rivieri Dur., a tropical member of Araceae, was successfully cultured to initiate callus tissue and adventitious shoots and roots. After this initial success, a long period elapsed with very little published in the area of monocot cultures. In the 1960's, tissue culture techniques became more refined and herbaceous dicot tissues were easily established in vitro. With these developments, interest in monocot tissue culture grew and more laboratories engaged in monocot cultures. Success came quickly following two developments as discussed by Sheridan (1975); the use of a new basal media with higher levels of mineral salts (Murashige and Skoog 1962; Linsmaier and Skoog 1965), and the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as an auxin. Within several years, all of the major monocot families had representative members successfully established in vitro (Sheridan 1975).

Lilium speciosum Thun. was the first lily to be cultured in vitro (Robb 1957). Explants were derived from cores of tissue bored out of fleshy bulb scales. Bulblets formed on explants derived from the base and middle regions of bulb scales. Bulblets were produced by such explants when obtained in the spring and autumn. Explants isolated during the summer and winter formed few or no bulblets. All explants were cultured on a modified White's medium (White 1943).

L. longiflorum was first established in culture by Sheridan (1968) on Linsmaier and Skoog's (1965) agar medium. Explants were derived

from the apex, grown on media with 2.0 mg/l indole-3-acetic acid (IAA) and formed callus within three weeks. Without IAA added to the medium callus was formed occasionally but only after formation of shoots and roots. Callus formed by explants on 2.0 mg/l IAA medium could be subcultured on agar media with or without IAA with no difference in growth rates. Growth rates in liquid shake cultures were greater than on agar media. Large numbers of plantlets could be induced in liquid shake cultures using callus masses. Plantlets arose as outgrowths of the large callus clumps. It was not necessary to disperse cells into single cell suspensions or small cellular clumps in order to obtain a high yield of plantlets. Kinetin (KIN) supplemented media did not increase callus growth rates in liquid cultures, and with concentrations of 1.0 mg/l or more, callus tissue turned brown and growth was inhibited.

Plantlet regeneration from Lilium regale tissue has been reported by Montezuma-de-Carvalho and Guimaraes (1974). Excised stamens served as explants and were placed on agar medium (Gautheret 1959) supplemented with 1.0 mg/l IAA and 2.0 mg/l kinetin. The filaments produced a visible callus at their cut ends after three weeks. Buds were evident after five weeks. The authors related their ease of obtaining plantlets to the propensity of some lilies (including L. regale) to form bulbils underground and at their leaf axils.

A more difficult group of monocots to culture in vitro have been the daylilies, Hemerocallis sp. The first Hemerocallis tissue culture was reported by Mullin (1970) who was able to establish callus tissue from tuberized roots. Organogenesis, however, was not induced. Chen and Holden (1972) reported induction of shoots and roots from daylily callus. Petals were dissected from flower buds (1-2cm in length) and placed on

a 6.0 mg/l naphthaleneacetic acid (NAA) agar medium. Adventitious roots emerged from approximately 10% of the explants. The rooted explants were then subcultured on a 5.0 mg/l 2,4-D medium to induce callus formation. Callus was formed by the roots at their point of contact with the medium. Plantlet regeneration occurred when callus tissue was transferred to a medium containing 1.0 mg/l 2,4-D and 1.0 mg/l kinetin. The conditions and media components necessary for maximizing plantlet production were not explored.

Heuser and Apps (1976) obtained callus directly from flower petal explants using a medium containing 1.0 mg/l 2,4-D and 1.0 mg/l kinetin. (As indicated above, this medium was used by Chen and Holden (1972) for the induction of organogenesis.) The callus was yellowish-green in color and appeared to develop meristematic protuberances. Plantlet formation occurred when the callus was transferred to a 1.0 mg/l kinetin medium lacking 2,4-D, but with IAA added at 0.5 mg/l. No mention was made of the relative frequency of callus tissue formation and plantlet regeneration. Little progress was made using scape explants since they formed a short-lived callus that did not survive subculturing. Liquid shake cultures were used to increase the amount of callus, but no mention was made of the rate and characteristics of this phase of callus growth.

Commercial production of daylilies using <u>in vitro</u> propagation techniques is presently in operation at Oglesby Nursery, Inc., Hollywood, Florida. Explants are obtained from the meristem area associated with the crown. Agar media (Murashige and Skoog 1962) supplemented with 0.2 mg/l IAA, 80 mg/l adenine, and 25 mg/l 2iP (N_6-(2-isopentenyl)-adenine) is used to induce explant growth and plantlet production. After an initial growing period, 4 to 6 weeks, the explants begin to produce plantlets. Each explant is divided into equal plantlet containing parts; one portion goes

back to fresh plantlet producing media, and the other portions are placed on a rooting medium, containing high auxin, where the plantlets undergo root formation and further growth. Large numbers of plantlets can be obtained by this procedure on a commercial scale:

> In our program we have 6 groups of tubes, 100 tubes in each group on a 2 x 12 foot shelf. 100 tubes are ready each week for division. From the divisions 100 tubes are put back on the shelf for the crop 6 weeks from the date. The remainder are cut in doubles and put in mason jars for rooting. This will provide 500 double plantlets each week.

While this approach to propagation is vastly superior to standard nursery practices, it was found that not all daylily varieties were equally productive. To achieve optimal plantlet regeneration, not only must the plantlet regenerating potential of the various tissues available for culture be assessed, but the interactions of the various plant hormones and growth regulators used to induce organogenesis must be thoroughly investigated.

Within the genus Hippeastrum, several reports have been published describing success regenerating plantlets from H. hybridum explants. Mii et al (1974) cultured excised bulb scales on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with NAA and kinetin. Explants developed buds, roots, or both depending upon the NAA concentration. Kinetin was toxic at high concentrations and had little influence on organogenesis. Oyamada (1974) reported culturing bulb scale explants on filter paper supplied with MS nutrient solution. Adventive buds were obtained when 1-2 ppm 6-benzylaminopurine (BA) with 5-10 ppm NAA was added to the nutrient solution. Hussey (1975) reported success regenerating plantlets directly from explants derived from the bulbs and inflorescence stem tissue on MS agar medium containing 2.0 parts 10^{-6} NAA. The explants did not respond to 2,4-D or IAA supplemented media.

Protoplasts have been isolated from Hippeastrum flower petals, as reported by Wagner et al (1978). Emphasis was placed on the ease of obtaining protoplasts and their use in programs of cell biology instruction and research. No mention was made of attempts at regenerating plantlets from the isolated protoplasts of Hippeastrum.

Skoog and Miller (1957) demonstrated that kinetin, in conjunction with IAA was necessary for growth of tobacco pith in vitro. By manipulating the auxin-cytokinin ratio, organogenesis could be controlled; tobacco pith tissue could be induced to form roots, shoots, or callus. These principles are of tremendous importance in establishing working methods for propagating plants using tissue culture methods. If rapidly dividing callus tissue can be produced and maintained in culture, then large amounts of uniform undifferentiated inoculum may be used to induce the regeneration of plantlets by manipulating the auxin-cytokinin ratio. This in vitro method of propagation would be far superior to standard propagation techniques because of the potentially unlimited number of clonal individuals produced.

Plants within the genera Lilium, Hemerocallis, and Hippeastrum are currently propagated horticulturally by the following methods (Bailey 1914, Benzinger 1968, and Hudson and Kester 1975):

Lilium:

a. Seeds: Flowering size bulb is produced in about four years.

b. Scales: Bulb scales broken off the mother bulb soon after the flowers have faded will yield bulblets when planted in a suitable rooting medium. Flowering size is reached in 3-4 years.

c. Bulbils: Some species produce small organs called bulbils at the leaf axils. These can be detached, treated as seeds, and grown to mature flowering size in two years.

d. Natural Separation: Bulbs may be dug every 4-5 years and the mother bulb separated from the surrounding bulbs and bulblets.

Bulbs and bulblets should be planted and handled according to their size and maturity.

Hemerocallis:

a. Seeds: Seedlings grow to flowering maturity in two years.

b. Natural Division: Clumps are dug up every 3-5 years and individual ramets (fans) are split off and planted separately.

c. Artificial Division: Mature plants are cut through the crown area to induce ramet formation. After 45 days, when the new ramets have emerged, the separation is completed. Plants should be gibberelin treated to promote rapid growth. Potentially, 128 plants can be produced from one plant in about one year, with an additional 2 years required for flowering maturity.

d. Proliferations: Some varieties produce plantlets along the scape. These may be separated from the scape and rooted to produce additional plants.

Hippeastrum:

a. Seeds: Requires 5 years growth until flowering maturity.

b. Offsets: Offsets separated from the mother bulb every five years. Approximately 3-4 years are required to reach flowering maturity.

c. Bulb Cuttings: Bulbs are cut vertically through the basal plate into 8-10 segments. Each segment may be cut between the 3rd or 4th bulb scale, to include a segment of the basal plate. These pieces are planted in a rooting media. Bulblets with roots develop within weeks. Bulblets are separated and transferred to soil to continue development.

MATERIALS AND GENERAL METHODS

Explants were surface sterilized for 10 minutes in a 5.25% sodium hypochlorite solution containing a few drops of Tween 20 as a wetting agent. Explants were then rinsed three times with 300 ml portions of sterile water for a total of ten minutes.

A modified Linsmaier and Skoog (1965) medium was used for all cultures. A listing of media components and abbreviations is contained in Appendix Table 1; IAA, KIN, and 2,4-D were added as indicated. The media were autoclaved for twenty minutes at 121°C and poured into sterile glass petri plates (100 x 15 mm) and allowed to solidify. Each petri plate contained approximately 40 ml of medium. All manipulations of plant tissue were performed under a laminar flow hood (Environmental Air Control, Inc., 747 Bowman Ave., Hagerstown, Maryland, 21740).

Explants were placed on the appropriate media after surface sterilization and incubated at 25°C in the dark. Light cultures were incubated at 25°C with a light bank of eight fluorescent tubes (24T12 high output; F24T12/CW/HO; cool white) with a 16/8 hour light-dark cycle. Cultures were placed on a shelf 12 inches below the light bulbs.

Tissue was obtained from various plant parts in order to determine a suitable explant source for the establishment of long-term cultures. The primary goals were to obtain sterile callus tissue capable of a relatively rapid growth rate with a high degree of uniformity, and to maintain tissue which is capable of organogenesis. Plant parts used to obtain explants and their general responses in culture are given in Appendix Table 2.

Lilium. The immature leaves associated with the apex of dormant bulbs provided excellent explants. Bulbs were purchased in the fall and stored (usually two months) in a refrigerator until experimentation commenced. Bulbs were prepared for isolation by stripping off the outer bulb scales until only scales tightly packed around the apex remained. The basal plate was trimmed to remove excess tissue and debris; this unit was surface sterilized. Following the rinse, the remainder of the bulb scales were plucked away to reveal the quiescent apex, stem, and associated immature etiolated leaves. These tissues needed no surface sterilization - they are sterile naturally within a healthy bulb. Therefore, these tissues could be excised free from the bulb remnant and immediately placed on the culture medium. In this way, superb explants could be obtained, since these tissues were free of damage from the surface sterilizing agent.

The number of leaf explants obtained from a single bulb depended on the bulb size, condition, and relative maturity. As few as six were obtained, in some cases, or as many as 34 explants could be plucked from a single bulb. A decrease in size and weight of explants, from about 17 mm in length weighing 30 mg, to about 5 mm in length weighing 2 mg, was noted while progressing from the outer explants to the internal explants. The smaller explants, in general, responded more slowly and less vigorously to culture conditions than larger explants. Contamination was essentially absent, and necrosis was minimal.

Hemerocallis. Young scape and flower bud tissues provided the only acceptable explants. Immature scapes (of various lengths) were selected and the succulent upper 3.5 inches (exceptions stated as they occurred) of the scape removed and immediately surface sterilized. Each scape was cut cross-sectionally into 3-5 mm segments and placed on appropriate media.

The flower buds were cut free from the scape and surface sterilized separately. Tissue necrosis, slow growth rates, contamination, and variation from scape to scape were problems that were encountered.

Hippeastrum. Tissue derived from the inflorescence stem within a dormant bulb proved to be an excellent tissue source. The bulb scales were excised and carefully removed until the dormant inflorescence stem and flower bud cluster was revealed. The inflorescence stem and flower bud cluster was pulled from the bulb and surface sterilized. Explants were obtained by first cutting the inflorescence stem base away (the sterilizing agent soaks into this area and kills this tissue) and then cutting cross-sectional slices (parallel to the base) every 5 mm. These explants were immediately placed on the culture medium. The number of explants isolated per scape ranged approximately from five to twenty five. Explants were comparable and free of contamination, but excessive tissue necrosis was often a problem.

Parent plant material used in this study was obtained from several sources. Lilium regale was used throughout this study to represent the genus Lilium. Regal lilies were purchased from Geo. W. Park Seed Co., Inc., Greenwood, South Carolina 29647 ("Regale Improved", catalog #8707). Hemerocallis varieties "Bitsy", "Queen of May", "Lavender Lady", "Emperor's Robe", and other daylilies were donated by Fred Benzinger of Benzinger Greenhouses, Route 1, Box 259, Ruckersville, Virginia 22968. Hippeastrum varieties were purchased from Geo. W. Park Seed Co., Inc., and Leo Berbee Bulb Co., Inc., Box 370, Marysville, Ohio 43040. The varieties of amaryllis bulbs purchased included Royal Dutch Hybrid "Sweet Seventeen" from Parks Seed Co., and "Fantastica" from Berbee Bulb Co.

The influence of the auxin-cytokinin ration upon the morphogenetic expression of explants in vitro was investigated. The specific methods used for these experiments are included in the Experimental Procedures and Results section under the individual species.

EXPERIMENTAL PROCEDURES AND RESULTS

Lilium regale:

Explants placed on agar medium undergo several changes in a few days. Visually uniform explants demonstrate physiological variation by responding differently to the same culture medium. A few explants develop a brown necrotic area at the broken edge of the base of the explant, while the majority of the explants do not produce this wound response. Most explants curl inward at their base as a result of differential enlargement and division and begin to elongate. Over a period of two to three weeks, elongation decreases and swelling at the base of the explant increases. These explants are capable of rapid growth, organ formation, and callus production. In general, the smaller explants, which were excised near the apex, do not respond as vigorously as larger explants. The majority of the smaller explants do not develop beyond a slight curling and swelling at the base.

Explants respond to basal media (without exogenous growth regulators) by producing bulblets, callus tissue, and swelling of the basal area. Considerable variation in the pattern of development was obtained using these explants. The most predominant pattern involves an explant producing one or two bulblets at the basal region with little or no callus tissue or swelling. However, some explants produce bulblets with swelling and callus production, while others form callus only with swelling in the basal region. Figure 1 illustrates this variation in response.

FIGURE 1: Variation of response by *Lilium regale* immature leaf explants cultured on basal medium.

A typical example of one of the patterns of development is as follows. A bulb was dissected to yield 29 explants; these explants were placed on a basal medium. The explants were arranged and numbered in order of excision. As stated earlier, the size of the explants progressively decreases (explant #1 was the largest). After three weeks, the first 11 explants produced nine bulblets; one of these produced two bulblets, 3 produced none, and 7 produced one bulblet each. Beyond the 11th explant, no bulblets were formed and only a few proliferated to produce centers for potential formation of bulblets. Some swelling of the basal region was evident in most explants, but the degree of swelling varied. Three very elongated explants (#16, 17, and 19) produced large amounts of callus tissue with little swelling of the basal area. The variation in responses could be the result of either inherent biological variation, or an artifact produced by variations in the explant excision procedure.

The extent of variation in response to basal medium caused by injury during the isolation process was assessed. Explants obtained according to the previously described method, had additional injury incurred by splitting the basal area. The frequency of bulblet production by these explants was not above that of normal explants cultured on a basal medium. Therefore, despite damage by splitting, explants responded normally to basal medium. This suggests the isolation technique, which incurs damage to the explant, would not cause variations in the growth of the explants.

The response of split explants is essentially that of an intact explant, as the following experiment demonstrates. Explants from a single bulb were obtained in the usual manner. Then, every other explant was

split vertically into two identical halves. Therefore, whole intact explants could be compared to half explants. All of the resulting tissues were cultured (in the manner outlined in Appendix Table 3) on basal, ½L, and 2,4-D media. Whole explants and split explants were cultured in such a way that the response of split explants could be compared to whole explants, and the influence of the culture media on both types of explants could also be determined. The results indicate that bulblet production was in most cases the same for half a split explant as it is for a whole explant on the same medium. As indicated by preliminary experiments, bulblet production is influenced by the medium; 2,4-D medium inhibits bulblet production, while ½L medium slightly increases bulblet production in comparison to basal medium. Larger explants produce more bulblets than smaller explants cultured on basal medium. Therefore, variation in bulblet production appears to be an interaction between the individual explant's capacity for bulblet formation and the influence of the medium. The variation does not arise as an artifact of the isolation technique.

The growth rate of explants cultured in the dark on ½L, 2,4-D, and 23 media was determined by periodically measuring fresh weights. It was decided to use 2,4-D as standard medium in subsequent experiments designed to promote undifferentiated explant growth. ½L medium also supported excellent explant growth, while 23 medium did not.

Kinetin, IAA, and 2,4-D not only influence the growth of explants, but also direct the potential organogenesis by explants. For example, an explant on ½L medium (2.0 mg/l IAA and 0.2 mg/l kinetin) produced up to 7 bulblets, while a total of 17 explants produced 44 bulblets for an average of 2.6 bulblets/explant. Explants growing on 2,4-D media rarely produced bulblets; growth occurred in the form of characteristic callus tissue.

Callus proliferation first appeared as a nodule of yellowish cellular growth and often had a loosely friable consistency. Callus, produced on only a few $\frac{1}{2}$L or basal medium explants, was not nodular and had a lighter yellow color.

Since standard 2,4-D medium (1.0 mg/l 2,4-D) initiated callus growth in the absence of organogenesis, an optimum concentration of 2,4-D was sought for best callus growth. Callus tissue was selected from existing stock cultures that had been maintained on standard 2,4-D medium. A total of 36 small pieces of callus tissue were selected for uniformity and placed on standard 2,4-D medium in order to allow time to detect contamination and provide an additional opportunity for selection for uniformity. The clumps were arranged into six groups, each group having six callus clumps. Increases in fresh weight were determined for each clump at regular intervals (4-6 days). After 18 days of monitoring growth on standard 2,4-D medium, each group was transferred to a new medium containing selected (0.5, 1.0, 3.0, 5.0, 7.0, and 10.0 mg/l) experimental concentrations of 2,4-D. For convenience, each group was referred to by the concentration of 2,4-D (mg/l) contained in the experimental medium upon which they were transferred on day 18. Increases in fresh weight were measured for an additional 20 days following this transfer to experimental media. The data are presented in Appendix Tables 4 and 5, and Appendix Figures 1 and 2.

The data shows the rate of growth for each group, while on standard 2,4-D medium, varied slightly and after the transfer of each group to their experimental media, growth rates slowly decline in all concentrations above 1.0 mg/l. The standard concentration of 2,4-D, 1.0 mg/l, is not an inhibitory concentration. It appears that it may not be the optimal concentration, however, as the 0.5 group growth rate on its experimental medium appeared to be greater than the 1.0 group.

The influence of the auxin-cytokinin ratio on L. regale explants was investigated. Explants were excised and placed on media containing varying concentrations of IAA and kinetin, as suggested by Skoog and Miller (1957). Three levels of IAA (0.2, 1.0, or 2.0 mg/l) in all combinations with three levels of kinetin (0.02, 0.2, or 1.0 mg/l) were added to the basal medium, thereby creating nine media types with differing auxin-cytokinin ratios. In addition, explants were also grown on basal medium (no growth regulators), ½L (0.2 mg/l kinetin with 2.0 mg/l IAA), and 2,4-D medium (1.0 mg/l 2,4-D) to serve as controls and provide a basis for comparison.

Explants were derived from four bulbs and arranged on the media so that the distribution of explants was equitable and comparable. Three bulbs were used as a source of explants on the nine IAA-kinetin media. Explants from one bulb were placed in an ordered sequence on the three media types of one kinetin level, beginning with the lowest IAA concentration and ending with the highest. In this way, explants within each level of kinetin were derived from the same bulb and were, therefore, comparable. Explants from the fourth bulb were cycled on basal, ½L, and 2,4-D media, in that order. Additional explants were obtained by sectioning the naked stem of the bulb into 3-5 mm cross-sectional wafers. Each resulting explant was cut into four eqivalent pie-shaped sections. These stem tissue explants were distributed randomly on the same three media types used to culture the immature leaf explants from that bulb.

Bulblet production, which occurred within 3-4 weeks, was recorded and summarized in Table 1.

TABLE 1

BULBLET FORMATION OF L. regale EXPLANTS ON IAA-KIN INTERACTION MEDIA

Media type (Conc. in mg/l)		Observations				
KIN	IAA	Bulblets per Leaf explant	Aver.	Bulblets per Stem explant	Aver.	Callus
0.02	0.2	19/10	1.9	15/7	2.1	+
0.02	1.0	11/11	1.0	1/6	0.2	+++
0.02	2.0	15/8	1.9	6/6	1.0	+++
0.2	0.2	13/15	0.9	23/8	2.9	−
0.2	1.0	9/12	1.0	3/2	1.5	+
0.2	2.0	9/10	0.9	25/12	2.1	++
1.0	0.2	23/11	2.1	10/9	1.1	−
1.0	1.0	16/13	1.2	21/12	1.7	−
1.0	2.0	13/8	1.6	15/6	2.5	+
Basal medium		24/18	1.3	20/9	2.1	−
½L medium		26/10	2.6	23/5	4.6	−
2,4-D medium		0/4	0	0/4	0	+++

The average number of bulblets produced per explant ranged from 0.9 to 2.1 on the nine IAA-KIN interaction media types. It was concluded, therefore, that the average number of bulblets produced per explant was relatively low, and organogenesis was not greatly influenced by manipulations of the auxin-cytokinin ratio. However, it is also possible that the concentrations of IAA and kinetin selected were not sufficiently extreme to produce more obvious responses. It is interesting to note that the average number of bulblets produced by the stem sections were, in general, slightly higher than the leaves. Therefore, the stem sections can differentiate bulblets as well as, or possibly better than, the leaf explants.

The $\frac{1}{2}$L medium (Appendix Table 1) includes IAA-2.0 mg/l and kinetin-0.2 mg/l. The replicate treatment (IAA-KIN interaction medium) indicates considerable variation in the capacity for bulblet formation between comparable explants. The following experiment explores this variation.

Explants excised from one bulb were placed in sequence on basal, $\frac{1}{2}$L, and 2,4-D media, in that order. Representative observations of growth bulblet production are presented in Table 2. The development of explants on basal medium was relatively uniform - an average of one bulblet per explant with some necrosis and a small amount of callus proliferation. Development on $\frac{1}{2}$L medium suggested a greater range of variation. Certain explants produced 3-4 bulblets, some produced one or two, while other explants produced none. The amount of swelling at the base varied widely among explants, and distinct callus was produced from only one explant. The development on 2,4-D medium was fairly consistent with explants producing callus and varying degrees of swelling at the base.

TABLE 2

THE GROWTH AND DIFFERENTIATION OF BULBLETS BY L. regale EXPLANTS ON BASAL, ½L, OR 2,4-D MEDIA

Explant	Number	Observations
Basal	1	one bulblet
	2	one bulblet
	3	one bulblet
	4	one bulblet
	5	one bulblet
	6	one bulblet
		Note: Upper part of individual explants necrotic with little swelling.
½L	1	greatly swollen, 3-4 bulblets
	2	greatly swollen, 3-4 bulblets
	3	swollen, no organization
	4	some swelling, one bulblet
	5	some swelling, no organization
	6	little swelling, no organization
2,4-D	1	good nodular callus growth, root hairs present but no visible roots
	3	one root, good swelling
	4, 5, and 6	swollen at the base, no distinct callus, no organization

Basal medium, having no exogenous growth regulators, promotes normal growth and elongation of the explants; bulblet formation and callus proliferation occurs at a low frequency per explant. 2,4-D medium, on the other hand, inhibits organogenesis, but promotes growth in the form of callus tissue. $\frac{1}{2}$L medium, having IAA and kinetin, promotes more rapid growth than basal medium. The auxin-cytokinin ratio, however, does not have an observable influence on the expression of morphogenetic potential. Bulblet formation and callus proliferation seem to occur with a greater degree of variation among the explants in response to $\frac{1}{2}$L medium. This suggests that $\frac{1}{2}$L medium promotes growth, the extent and direction of which is determined by the potential expression of individual explants.

Callus tissue that was formed on either basal or $\frac{1}{2}$L medium was easily subcultured, and maintained a rapid growth rate. Bulblets were produced intermittently and could easily be separated from the callus and allowed to further develop in the light. A high yield of plantlets is desirable for an in vitro propagation system, therefore, the bulblet forming potential of this callus tissue was investigated. Isolated callus has been reported to be induced into plantlet regeneration by manipulating the auxin-cytokinin ratio (Narayanaswamy 1976); a high kinetin-low auxin medium induces shoot morphogenesis. In this investigation, L. regale callus was transferred to an IAA-KIN interaction series to screen for a medium which would induce a high frequency of bulblets.

Isolated callus derived from explants cultured on basal medium was subcultured several months and an abundance of callus was produced. Comparable callus clumps were selected from this stock tissue and transferred to media containing all combinations of selected concentrations of IAA and KIN. Bulblet production was recorded after 12 weeks (Table 3).

TABLE 3

BULBLET PRODUCTION OF L. regale CALLUS IN RESPONSE TO IAA-KIN INTERACTION MEDIA

Media type (Conc. in mg/l)		Observations (after 12 weeks)	
KIN	IAA	Bulblets/clumps of callus	Average
0	0	82/18	4.6
0	0.2	76/13	5.8
0	1.0	54/15	3.6
0.02	0	71/14	5.1
0.02	0.2	62/13	4.8
0.02	1.0	39/13	3.0
0.02	2.0	56/14	4.0
0.2	0	86/15	5.7
0.2	0.2	39/15	2.6
0.2	1.0	70/20	3.5
0.2	2.0	92/15	6.1
1.0	0.2	102/22	4.6
1.0	1.0	74/12	6.2
1.0	2.0	61/13	4.7

The results indicate a range of average bulblet production per callus clump from 2.6 to 6.2. Analysis of the data indicated that large variations in bulblet production (of callus clumps cultured on the same medium) resulted in a lack of significant difference between the sets of data. Therefore, bulblet production by callus tissue appears to be unaffected by manipulations of the auxin-cytokinin ratio in the medium.

Unfortunately, callus that initially develops in response to 2,4-D medium does not readily differentiate bulblets. 2,4-D callus transferred to basal medium was not induced to form bulblets. Therefore, it seems 2,4-D callus was irreversibly inhibited in short-term morphogenetic potential. However, more experimentation is necessary before such a conclusion could be substantiated.

Hemerocallis:

Preliminary experiments with Hemerocallis explants indicated the presence of morphogenetic potential since shoot production was obtained in initial attempts to culture scape explants. Only on two occasions were shoots produced in these exploratory experiments. Pedicel tissue isolated from a scape of "Emperor's Robe" produced shoots after several months in culture. At the time of isolation, the scape was approaching flowering maturity with the leading floral bud gaining color and near opening. The subtending floral buds ranged in size from several centimeters to about 1 cm in length. Pedicel tissue from each floral bud was surface sterilized, isolated, and cultured on $\frac{1}{2}$L, 2,4-D, or 23 media. The only tissue that produced shoots was the tissue derived from the pedicel of the smallest (about 1 cm) floral bud cultured on $\frac{1}{2}$L medium. This tissue produced one explant which resulted in swollen root-like structures accompanied by a small amount of callus tissue. After three months, the callus tissue developed organized structures which were separated from the original explant. One month later, a shoot emerged from this isolated callus mass. The callus and its shoot was then placed in the light and additional roots developed from the plantlet after a few weeks. This plantlet was established in soil and is presently adult size with four to five ramets (fans).

Plantlets also arose from scape and perianth tissue isolated from the scape of a "Benzinger" hybrid daylily. Scape tissue was isolated in the manner described earlier, except before being placed on the medium, each explant was split longitudinally (along the vertical axis of the scape) to yield two identical explants. One of each of the identical explants was placed on 2,4-D medium, while the other was placed on $\frac{1}{2}$L

medium. Therefore, differences in morphogenetic potential of the explants would be the result of differences in the growth regulators in the two media, and not as a result of position on the scape. Explants on 2,4-D medium developed only roots from the ground tissue within 3 weeks, while explants on $\frac{1}{2}$L medium developed only shoots within 4 weeks. Explants on 2,4-D medium, regardless of position (upper vs lower) on the scape, uniformly developed roots. This uniformity of response was not observed using explants on $\frac{1}{2}$L medium; explants derived from scape tissue close to the floral buds produced shoots, while explants derived from the basal region of the scape became necrotic.

The successful production of shoots from daylily explants became more frequent in subsequent isolations of scape tissue. Further experimentation was limited to one daylily variety ("Queen of May"); a number of parent plants were available, and were easily induced to flower. Therefore, a large number of suitable uniform explants could be obtained. Also, preliminary results indicated suitable morphogenetic potentials using this variety. A procedure for the induction of flowering of parent plants is given in Appendix Table 8.

The initial attempts to culture "Queen of May" scape tissue involved four types of media: basal, $\frac{1}{2}$L, 2,4-D, and Apps (Appendix Table 1). The first scape was $8\frac{1}{4}$ inches in length at the time of isolation, and yielded 45 explants. Each explant was divided longitudinally into quarters to yield four identical pie-shaped wedges of tissue. One tissue piece from each explant was placed on each type of medium.

The tissue became swollen and elongated within 1-2 weeks on all media types. Within 2-3 weeks a few sections developed roots in response to $\frac{1}{2}$L medium. This did not occur on the other media types at this time.

In general, those sections which developed roots on ½L medium were originally isolated from the upper portion of the scape.

Within 5-6 weeks, a shoot had emerged from one of the tissue explants on ½L medium. Most explants on 2,4-D medium developed roots regardless of position on the scape before isolation - a pattern not reflected by explants on ½L medium. Also, the roots of 2,4-D cultures tended to be thicker than the roots of ½L cultures. Tissue on basal medium showed no signs of developing beyond the swelling stage; on Apps medium tissues failed to grow and became mushy in most cases.

Attempts to stimulate shoot production were made using four tissue pieces from each of the four media treatments employed in the previous experiment. Representive tissues were selected and transferred to a high kinetin medium containing 1.0 mg/l kinetin and 0.5 mg/l IAA. This level of kinetin was selected because other investigations (Chen and Holden 1972, Apps and Heuser 1976) have reported success initiating shoots with high kinetin medium. Stoutemyer (1976) summarized recent success using cytokinins for plantlet regeneration of daylilies.

After an additional 10 weeks on high kinetin medium, shoot primordia had developed on a few explants. Of the tissues that remained on 2,4-D medium, none had developed shoot primordia, but a unique "root-callus" growth was produced. This "root-callus" is best described as abnormal thickened roots interspersed with amorphous clumps of tissue with an overall light yellow coloration. Of the four tissues from 2,4-D that were transferred to high kinetin, one developed shoot primordia. Thickened roots, but without callus, also developed from these four tissues. Tissues remaining on ½L medium developed additional roots, and a few shoot primordia, while tissues on high kinetin developed additional roots and a few shoot primordia. Tissue remaining on Apps medium (high

kinetin) also developed shoot primordia.

The floral buds from the "Queen of May" scape used in the previous experiment were also cultured according to the procedure described by Heuser and Apps (1976), who reported success using petals dissected from floral buds approximately 1 cm in length. In this experiment, the buds were cultured by longitudinally splitting the buds open along the furl of the sepals, thereby including all bud tissue in the culture. These tissues, placed on Apps medium, became necrotic with only a few explants remaining viable. After 4 months in culture, a small amount of callus developed and a few shoot primordia were produced at the base of each viable bud. This culture was transferred to the light-bank incubator to allow additional development of the shoots. After 7-8 weeks, a small area of several minute shoots emerged from one of the buds, while the other bud had two densely packed green areas where very small organized structures were visible. These structures could not be distinguished as shoots or roots in these initial observations. These results closely agreed with the work reported by Heuser and Apps (1976).

The next experiment was conducted using a scape from "Queen of May" that was $8\frac{1}{2}$ inches in length and yielded 42 explants. Explants were excised without subdivision and placed on four media types - basal, $\frac{1}{2}$L, 2,4-D, and Apps. A similar pattern of growth and morphogenesis, as suggested in the previous experiment, was confirmed using these cultures. Explants on basal medium, as a result of the absence of growth regulators, became necrotic. On $\frac{1}{2}$L medium, roots developed rapidly (2-3 weeks) from explants derived from the upper portion of the scape and only one explant developed a shoot. On 2,4-D medium, a few explants became necrotic, while the majority of the explants developed the characteristic "root-callus" growth, normally associated with 2,4-D cultures. This is con-

trary to the results reported by Heuser and Apps (1976), where scape tissue produced a compact white callus that did not survive subculturing. Floral buds were not cultured in subsequent experiments since scape explants had demonstrated a more promising morphogenetic potential in experimental cultures. Additional experiments were designed to elucidate the factors responsible for inducing shoot formation using scape explants.

As previously stated, the auxin-cytokinin ratio has been shown to direct the morphogenetic potential of tobacco explants (Skoog and Miller 1957) in vitro. An additional "Queen of May" scape was isolated and cultured on media containing various concentrations of IAA and kinetin to determine the influence of the auxin-cytokinin ratio on daylily scape explants. The scape used was approximately 13-15 inches in height. As a result of the increased length, this scape was cut every $3\frac{1}{2}$ inches starting from the top, to yield three (top, middle, and bottom) sections that were surface sterilized. After the final rinse, each section was cut into numbered sequential explants and placed in order on basal medium. One hundred forty-six explants were obtained in order from top to bottom of the original scape. The IAA-kinetin interaction media (Table 4) included three concentrations of IAA (0.2, 1.0, and 2.0 mg/l) and kinetin (0.02, 0.2, and 1.0 mg/l) based on concentrations suggested in the literature (Sheridan 1968, Chen and Holden 1972, and Heuser and Apps 1976). One sequential explant was transferred to each type of IAA-kinetin medium and repeated until explants were distributed in order on the fourteen media. This procedure was repeated until each medium had ten explants. Observations were recorded after 6 and 14 weeks. The data is represented in Table 4.

TABLE 4

RESULTS OF Hemerocallis cv. "Queen of May" SCAPE EXPLANTS ON IAA-KIN INTERACTION MEDIA

Media type (Conc. in mg/l)		Observations/no. of explants			
		Shoots		Roots	
KIN	IAA	6 wks.	14 wks.	6 wks.	14 wks.
0	0	0	0	0	0
0	0.2	0	1/1	2/1	N.C.
0	1.0	0	1/1	7/1	N.C.
0.02	0	0	0	0	0
0.02	0.2	0	0	0	0
0.02	1.0	0	3/1	5/2	N.C.
0.02	2.0	0	3/2	49/4	N.C.
0.2	0	0	0	0	0
0.2	0.2	0	0	0	0
0.2	1.0	0	1/1	3/1	N.C.
0.2	2.0	1/1	7/2	21/3	N.C.
1.0	0.2	8/1	20/3	0	3/2
1.0	1.0	1/1	N.C.	1/1	N.C.
1.0	2.0	1/1	4/2	12/1	N.C.

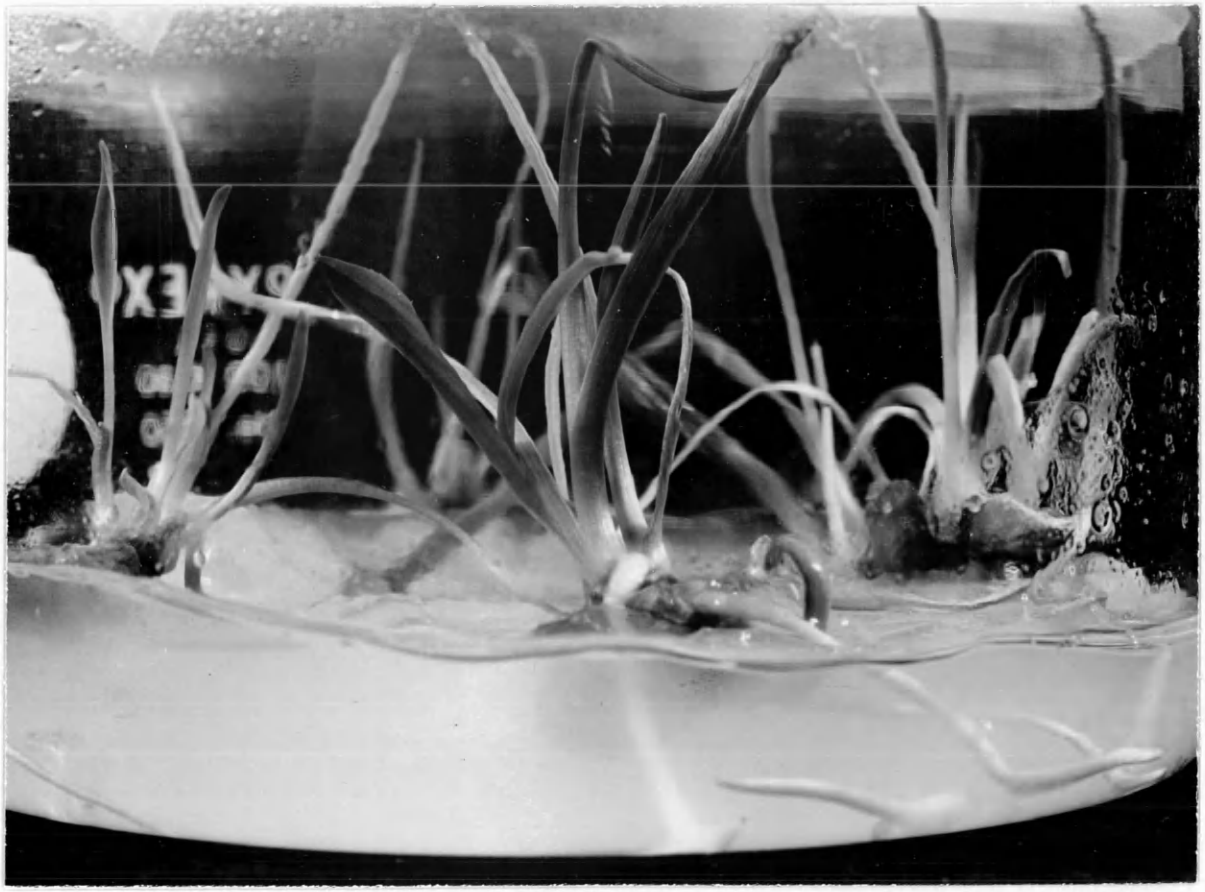
N.C. = No change

From the data, the following trends can be established. Essentially all explants in the approximate lower portion of the scape became necrotic; the one exception being an explant on IAA-2.0 KIN-1.0. The IAA-kinetin ratio was shown to exert a control over morphogenesis. The frequency of shoot initiation was highest at high levels (1.0 mg/l) of kinetin with a low level (0.2 mg/l) of IAA. Kinetin alone induced no organogenesis in this experiment, while IAA alone induced both root and shoot production at low frequencies. Note that at lower concentrations of both IAA and kinetin, little organogenesis occurred. The results also indicated rapid callus proliferation did not occurr in the presence of IAA or kinetin, or in any combination of the two growth regulators for daylily explants. As stated earlier, 2,4-D induces a unique "root-callus" growth. While 2,4-D has the capacity to induce callus growth, IAA or kinetin does not.

Additional isolations of "Queen of May" scape tissue were performed to confirm the response of scape explants to high IAA, high kinetin, $\frac{1}{2}$L, and 2,4-D media. Four scapes, ranging in height from $10\frac{1}{2}$ inches to $15\frac{1}{4}$ inches, were isolated according to the standard procedure. Appendix Tables 6 and 7 summarize the results obtained by culturing explants on the medium types mentioned. For convenience, each scape is considered an isolation and referred to by an isolation number.

Essentially, the morphogenetic pattern described earlier was confirmed. Shoots are induced on high kinetin medium associated with a low auxin level. However, shoots also developed in the presence of high kinetin without auxin in two cases, therefore, auxin was not essential for the initiation of shoots on high kinetin medium. Figure 2 illustrates shoot production by scape explants in response to high kinetin medium.

FIGURE 2: Shoot production by Hemerocallis cv. "Queen of May" scape explants in response to high kinetin medium (KIN-1.0 mg/l, IAA-0.2 mg/l).



Hippeastrum:

Explants were obtained by sectioning the inflorescence stem tissue within dormant bulbs; bulbs were refrigerated before experimentation began (usually 2 or more months). Initially, explants developed a red color, generally associated with the cut surfaces, within a week. Discoloration of the agar medium often occurred as the substance diffused into the medium. No attempt was made to characterize the nature of this reaction or the identity of the substance causing the red coloration.

Despite the general reddening, most explants showed signs of growth and proliferation. Explants developed what appeared to be meristematic lumps protruding from the ground tissue of the inflorescence stem. Development of these lumps was slow and occurred over a period of three to six weeks. Growth of the epidermal layer and subjacent cell layers occurred on some explants, seemingly independent of the other tissues of the explant. This area developed into an expanded white sheet of enlarged cells, not a callus. Bulblets were observed arising from both areas of growth.

Explants cultured on basal medium developed bulblets within 3 weeks in some cases, but usually within 5-8 weeks. In one isolation, a total of 42 bulblets were produced from the explants of one inflorescence stem. This inflorescence stem yielded a total of 26 explants, six of which produced bulblets. Four of these six explants were derived from the lower portion of the inflorescence stem - the stem tissue just above the point of attachment to the bulb. One bulblet-producing explant was derived from the middle portion of the stem tissue, and the other was derived from the tissue of the inflorescence stem at the base of the flower bud cluster. Table 5 summarizes the data obtained from this isolation.

TABLE 5

RESULTS OF *Hippeastrum* cv. "Sweet Seventeen" EXPLANTS CULTURED ON BASAL MEDIUM

Explant No.[1]	Observations	Final Yield (1 yr.)
1	necrotic	none
2	necrotic	none
3	5 bulblets (5-6 weeks)	9
4	3 bulblets (5-6 weeks) 1 bulblet (8 weeks)	4
5	2 bulblets (8 weeks)	2
6	7 bulblets (5-6 weeks)	12
7 - 10	surface callus only	none
11	1 bulblet (8 weeks) many callus bumps	12
12 - 24	necrotic	none
25	surface callus only	none
26	3 bulblets (3 weeks)	3

[1]Explants numbered according to original position within inflorescence stem tissue (low numbered explants explants originally in the lower, or basal, region).

Explants responded to 2,4-D medium by producing roots. Roots emerged from the ground tissue of the explant within 6-8 weeks and grew rapidly, often producing faciated roots and other abnormal root structures. A small number of bulblets were produced by 2,4-D cultured explants, but bulblet formation was delayed several months. Because of this strong inhibition of bulblet formation, 2,4-D medium was not included in subsequent cultures.

Since basal medium allowed bulblet production, the possibility of increasing the frequency of bulblet production and/or callus proliferation through manipulations of the auxin-cytokinin ratio was investigated. Explants were derived from two inflorescence stems from one Hippeastrum cv. "Fantastica" bulb. Since the stems were short, explants were sliced into halves and randomly distributed onto IAA-KIN interaction media. The results, presented in Table 6, were tabulated 8 weeks after isolation.

The results indicated that bulblet production occurred on nearly every IAA-KIN media type. Therefore, the auxin-cytokinin ratio does not appear to influence the morphogenetic potential of Hippeastrum cv. "Fantastica" explants. Roots were not produced by the explants. This pattern of development is remarkably similar to the pattern demonstrated by Lilium regale explants on IAA-KIN interaction media. The callus of Hippeastrum explants, in this experiment, was not friable and was not easily separated from the explants. The production of bulblets by Hippeastrum cv. "Fantastica" explants is illustrated in Figure 3. The explants shown are representative bulblet-producing explants selected from the IAA-KIN interaction experiment described above.

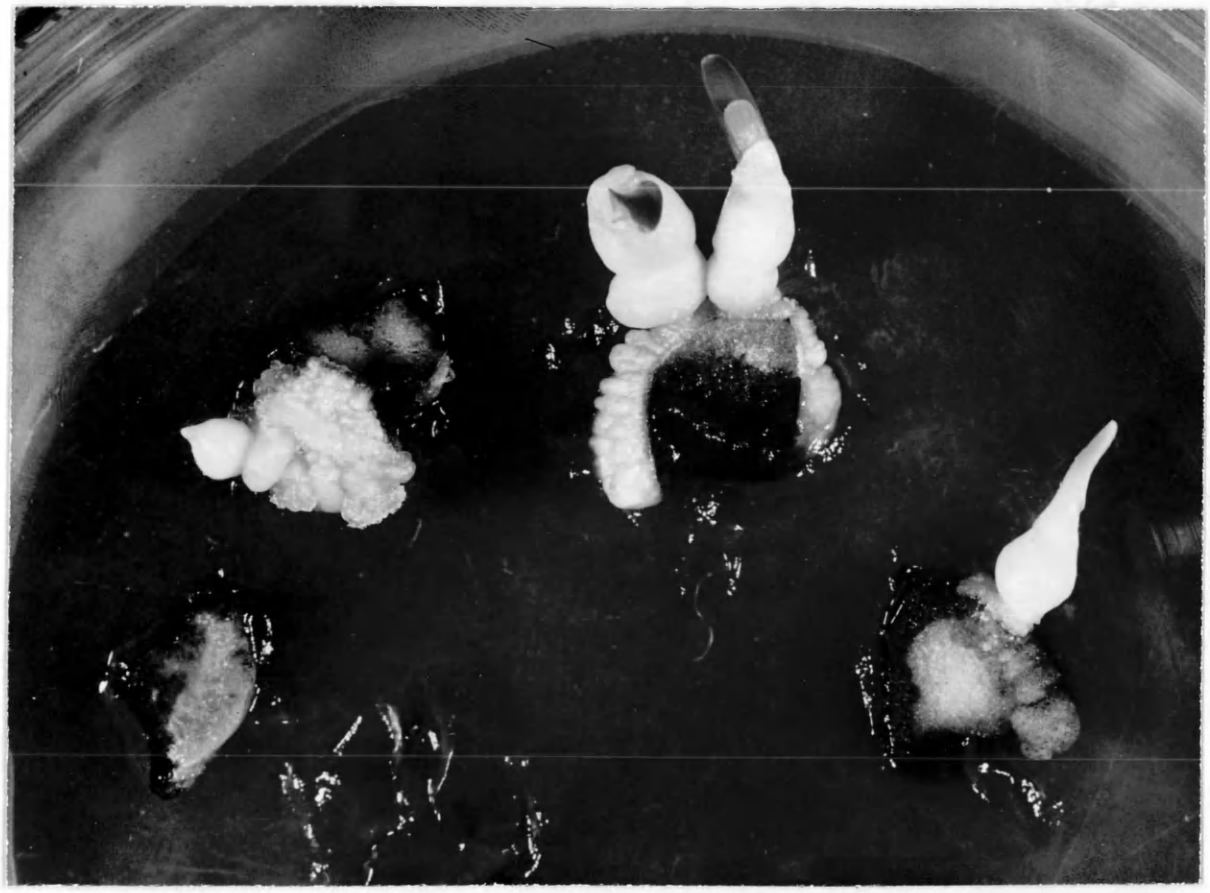
TABLE 6

RESULTS OF Hippeastrum cv. "Fantastica" EXPLANTS CULTURED ON IAA-KIN INTERACTION MEDIA

Media type (Conc. in mg/l)		Observations (after 8 weeks)		
KIN	IAA	Bulblets	Roots	Callus
0	0*	0	0	0
0	0.2	3	0	+++
0	1.0	3	0	+
0.02	0*	0	0	0
0.02	0.2	1	0	++
0.02	1.0	2	0	++
0.02	2.0	2	0	+
0.2	0	3	0	++
0.2	0.2	0	0	0
0.2	1.0	3	0	0
0.2	2.0	2	0	0
1.0	0.2	0	0	+++
1.0	1.0	0	0	0
1.0	2.0	2	0	0

*Tissue injury occurred during surface sterilization

FIGURE 3: Bulblet production by Hippeastrum cv. "Fantastica" inflorescence stem tissue explants in response to various IAA-KIN supplemented basal media.



Procedure for the establishment of plantlets in soil. Continued growth of shoots or bulblets and their subsequent isolation resulted in the development of a reliable method for establishing plantlets in soil. Initially, all cultures were incubated in the dark. As shoots or bulblets differentiated, the structures were isolated and transferred to sterile glass 8 oz. jars with air tight lids (or sterile 100 x 80 mm Pyrex No. 3250 vessels) containing basal medium. The containers with the isolated shoots or bulblets were sealed tight, to prevent contamination, and placed in the light-bank incubator (16 hr. light - 8 hr. dark). These cultures remained under these conditions for several months to allow full development of the plantlets, as would normally occur during a growing season.

After this phase of "summer", a "winter" was given to the cultures. The containers were removed from the light-bank incubator and placed in a growth chamber programmed for an 8 hour day of 55°F and a 16 hour night of 42°F. The containers remained in this growth chamber for two months.

Following the "winter" phase, the containers were opened and the plantlets removed. Prior to potting the plantlets in soil, care was taken to separate individual plantlets from one another and to wash off as much agar medium as possible. The plantlets were potted in soil and placed in the greenhouse. New growth emerged within 1-2 weeks.

CONCLUSIONS

Based on the data presented, Lilium regale explants demonstrated a tremendous potential for plantlet regeneration. Explants developed bulblets and callus on basal medium. In general, one bulblet was produced per explant in response to basal medium. IAA and kinetin supplemented media increased the average bulblet production per explant slightly, but also increased the variation of responses to the culture medium. This suggests that, while L. regale explants are capable of growth and organogenesis in response to basal medium, IAA and kinetin supplements to basal medium allow the inherent potential for bulblet production to be fully expressed, thereby increasing the range of explant response to the culture medium. It is suggested this is a reflection of the propensity for bulblet and bulbil production by L. regale bulbs in nature.

Manipulations of the auxin-cytokinin ratio, using IAA and kinetin, had no significant control over the expression of organogenic potential by L. regale explants. Bulblets were produced by explants cultured on IAA-KIN interaction media regardless of the auxin-cytokinin ratio; independent shoots or roots were never observed from such cultures. It is possible that the range of IAA and kinetin concentration selected were not sufficiently extreme. Since experimentation with other auxins or cytokinins was not within the scope of this investigation, it is not possible to predict L. regale explants would respond in a similar manner to auxin-cytokinin ratio manipulations using other growth regulators.

L. regale callus was produced by explants cultured on basal medium and IAA-KIN supplemented media. The callus was friable, easily subcultured for extended periods of time, and retained the potential for bulblet production. IAA-KIN ratio manipulations did not significantly influence the bulblet producing potential of *L. regale* callus. Callus initiated by explants cultured on standard 2,4-D medium was also friable and easily subcultured. However, the bulblet producing potential of 2,4-D callus was apparently irreversibly suppressed; 2,4-D initiated callus did not produce bulblets when transferred to basal medium. Growth of 2,4-D callus was suppressed by concentrations of 2,4-D above 1.0 mg/l.

The bulblet producing potential of *L. regale* cultures was impressive. One hundred bulblets could easily be obtained *in vitro* by explants derived from a single bulb within a few months. Greater numbers, up to 1,000, of bulblets could be obtained by subculturing callus and isolating bulblets as they developed.

Initial attempts at cultures of *Hemerocallis* tissues produced few positive results. However, subsequent attempts demonstrated that *Hemerocallis* explants derived from scape tissue possessed a good potential for shoot morphogenesis. Previous investigations did not use scape tissues with success (Chen and Holden 1972, Heuser and Apps 1976). Shoot morphogenesis was demonstrated to be the result of a high concentration of kinetin (1.0 mg/l) using *Hemerocallis* cv. "Queen of May" scape explants. Although a low concentration (0.2 mg/l) of IAA was included in the medium, high kinetin alone did induce shoot morphogenesis. Scape explants responded to high concentrations of IAA by producing roots. Therefore, unlike *L. regale*, *Hemerocallis* scape explants respond classically to manipulations of the auxin-cytokinin ratio *in vitro*. Unfortunately, a medium containing

IAA and kinetin capable of inducing callus proliferation from scape explants was not discovered. An unusual combination of abnormal roots with callus tissue interspersed was obtained from 2,4-D (1.0 mg/l) cultured explants. This tissue did not demonstrate a plantlet regenerating potential. However, an effective *in vitro* propagation method can be applied by culturing scape explants on high kinetin medium. Such a method could rapidly increase the number of daylilies of a given type.

Hippeastrum explants produced bulblets in response to basal medium. Callus proliferation did occur, but the resulting callus was not friable or easily separated from the explant. Like *L. regale*, *Hippeastrum* explants produced bulblets on all IAA-KIN supplemented media, regardless of the auxin-cytokinin ratio. In contrast, 2,4-D cultured explants initiated many roots. Occasionally, a few bulblets were produced by 2,4-D cultured explants, however, the frequency of bulblet production for basal medium cultured explants was greater. In one case, a total of 42 bulblets were produced by explants derived from a single inflorescence stem. These methods could be used effectively to propagate *Hippeastrum* varieties *in vitro*.

Plantlets and bulblets were easily established in soil by inducing dormancy. An artificial winter was administered to fully developed plantlets while still *in vitro*. After two months of "winter", the plantlets were removed from their culture containers, separated, washed, potted in soil, and placed in the greenhouse without injury or loss.

SUMMARY

In summary, Lilium regale, selected Hemerocallis and Hippeastrum varieties were successfully propagated in vitro using the following procedures:

L. regale: Explants derived from within quiescent bulbs (immature leaves and stem sections) developed callus and bulblets on basal medium in 3-4 weeks. ½L medium (0.2 mg/l kinetin, 2.0 mg/l IAA), in general, increased bulblet production. Callus was easily subcultured to yield additional bulblets. Potentially, approximately one hundred bulblets could be produced by explants from a single bulb. Bulblets quickly developed into plantlets in the light.

Hemerocallis: Explants derived from young scape tissue produced numerous shoots within 6 weeks when cultured on a high kinetin medium (1.0 mg/l kinetin, 0.2 mg/l IAA). The shoots rapidly developed into plantlets after the cultures were placed in the light.

Hippeastrum: Explants derived from the inflorescence stem within quiescent bulbs developed bulblets on basal medium and IAA and kinetin supplemented media within 4-8 weeks. Bulblets developed into plantlets when transferred to the light.

Plantlets were allowed to develop in vitro for several months in the light. Following a two month cold treatment, plantlets were established in soil.

APPENDIX TABLE 1

MEDIA COMPONENTS AND ABBREVIATIONS

Basal medium

Chemical:	Conc. (mg/l)
NH_4NO_3	825.0
$CaCl_2 \cdot 2H_2O$	220.0
KH_2PO_4	85.0
Na_2EDTA	18.6
$FeSO_4 \cdot 7H_2O$	13.9
$MgSO_4 \cdot 7H_2O$	185.0
KNO_3	950.0
H_3BO_3	3.1
$MnSO_4 \cdot H_2O$	8.5
$ZnSO_4 \cdot 7H_2O$	5.3
KI	0.41
$Na_2MoO_4 \cdot 2H_2O$	0.17
$CuSO_4 \cdot 5H_2O$	0.017
$CoCl_2 \cdot 6H_2O$	0.017
Thiamine HCl	0.4
myo-Inositol	100.0
Sucrose	30.0 gm/l
Agar	8.0 gm/l

23 medium

Chemical:	Conc. (mg/l)
$Ca(NO_3)_2 \cdot 4H_2O$	200.0
$MgSO_4 \cdot 7H_2O$	360.0
Na_2SO_4	200.0
KNO_3	80.0
KCl	66.0
$NaH_2PO_4 \cdot H_2O$	16.0
Ferric Citrate	10.0
$MnSO_4$	3.0
$CuSO_4 \cdot 5H_2O$	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.025
H_2SO_4 (S.G. 1.84)	0.0005
H_3BO_3	0.05
$ZnSO_4 \cdot 7H_2O$	0.5
Agar	8.0 gm/l
Sucrose	20.0 gm/l
NAA	0.5 mg/l
Coconut milk (Heated to 60°C, cooled and filtered)	100.0 ml

MEDIA ABBREVIATIONS

Abbreviation:	Components:
Basal	basal medium only
½L	basal medium plus IAA 2.0 mg/l, KIN 0.2 mg/l
2,4-D	basal medium plus 2,4-D 1.0 mg/l
Apps	basal medium plus 2,4-D 1.0 mg/l, KIN 1.0 mg/l

APPENDIX TABLE 2

PLANT PARTS USED TO OBTAIN EXPLANTS AND THEIR GENERAL RESPONSE IN CULTURE

Lilium:	Ease in Sterilization	Cell Growth and Proliferation	Developed Callus	Organo-genesis
bulb scales	+	−	−	−
immature leaves	+	+	+	+
apex	+	+	+	+
stem	+	+	+	+
Hemerocallis:				
tuberized roots	+	−	−	−
apex	−	−	−	−
anthers	+	−	−	−
scape	+	+	+	+
flower buds	+	+	−	+
Hippeastrum:				
scape	+	+	+	+
flower buds	+	+	−	+
apex	−	−	−	−

APPENDIX TABLE 3

BULBLET PRODUCTION IN RESPONSE TO SPLITTING AND CULTURE MEDIA BY L. regale EXPLANTS IN VITRO

		Culture media and bulblet production( )		
Explant no.[1]	Treatment	Basal	½L	2,4-D
1	none	1-whole(5)		
2	split	½2.....(3) ½2.....(2)		
3	none		3-whole(3)	
4	split	½4.....(1)	½4.....(3)	
5	none			5-whole(0)
6	split	½6.....(2)		½6.....(0)
7	none	7-whole(2)		
8	split	½8.....(1) ½8.....(2)		
9	none		9-whole(3)	
10	split	½10.....(0)	½10.....(3)	
11	none			11-whole(0)
12	split	½12.....(0)		½12.....(0)

[1]Explants numbered according to order of isolation from bulb (explant no.1 first to be isolated, etc.).

APPENDIX TABLE 4

AVERAGE FRESH WEIGHT INCREASE OF L. regale CALLUS ON VARIOUS 2,4-D MEDIA

	Average Fresh Weight in mgm					
	GROUPS					
Day	0.5	1.0	3.0	5.0	7.0	10.0
0	35.3	38.2	48.5	52.2	49.9	34.8
4	42.5	44.1	56.4	60.4	56.3	41.5
8	44.1	47.0	58.7	65.6	53.9	38.0
12	49.6	49.1	59.7	76.8	65.2	44.8
18[1]	56.6	54.0	65.0	91.4	69.8	49.7
22	64.0	58.6	69.2	105.1	81.4	52.3
26	69.6	62.7	76.9	114.8	86.8	54.1
30	76.9	66.9	79.4	124.4	90.4	58.0
34	83.9	73.8	82.8	130.1	92.7	59.1
38	97.1	79.8	83.7	134.6	96.0	60.6

[1]Callus tissues were transferred from standard 2,4-D medium to their respective experimental concentrations of 2,4-D after being weighed on day 18. Throughout the experiment, the groups of callus tissue were referred to by the concentration of 2,4-D (in mg/l) contained in their respective experimental 2,4-D media.

APPENDIX TABLE 5

CUMULATIVE GROWTH INDEX OF L. regale CALLUS ON VARIOUS 2,4-D MEDIA

	Cumulative Growth Index[1] GROUPS					
Day	0.5	1.0	3.0	5.0	7.0	10.0
0	–	–	–	–	–	–
4	1.20	1.15	1.16	1.16	1.13	1.19
8	1.25	1.23	1.21	1.26	1.08	1.09
12	1.40	1.28	1.23	1.47	1.31	1.29
18[2]	1.60	1.41	1.34	1.75	1.40	1.43
22	1.81	1.53	1.43	2.01	1.63	1.50
26	1.97	1.64	1.58	2.20	1.74	1.55
30	2.18	1.75	1.64	2.38	1.81	1.67
34	2.38	1.93	1.71	2.49	1.86	1.70
38	2.75	2.09	1.72	2.58	1.92	1.74

[1] Cumulative Growth Index = $\frac{\text{Initial fresh wt.}}{\text{Final fresh wt.}}$

[2] Callus tissues were transferred from standard 2,4-D medium to their respective experimental concentrations of 2,4-D after being weighed on day 18. Throughout the experiment, the groups of callus tissue were referred to by the concentration of 2,4-D (in mg/l) contained in their respective experimental 2,4-D media.

APPENDIX TABLE 6

RESULTS OF Hemerocallis cv. "Queen of May" ISOLATION No. 4

Scape height: $9\frac{1}{2}$ inches

Medium (Conc. in mg/l)	Observations[1]
KIN-1.0 IAA-0.2	#2 (5 shoots) #3 (4 shoots) #4 (10 shoots) (1 root) #6 (3 shoots)
KIN-0.02 IAA-2.0	#7 (1 root) #8 (7 roots)
2,4-D (standard)	#4 (4 roots) #5 (5 roots)

[1]Observations listed according to explant no. (explants numbered relative to original position within the scape - low numbered explants originally in the upper portion of the scape).

APPENDIX TABLE 7

RESULTS OF Hemerocallis cv. "Queen of May" ISOLATION No.'s 3, 5, and 6

	Observations[1]		
	Isolation No.3	Isolation No.5	Isolation No.6
Scape height:	$10\frac{1}{2}$ in.	$12\frac{1}{2}$ in.	$15\frac{1}{4}$ in.
Medium (Conc. in mg/l)			
KIN-1.0 IAA- 0	#1 (1 shoot) (2 roots)	No developments	#1 (5 shoots) (1 root)
KIN- 0 IAA-2.0	#4 (4 roots)	#1 (1 root) #2 (2 roots) #3 (25 roots) #4 (3 roots) #5 (3 roots)	No developments
KIN-1.0 IAA-0.2	#1 (6 shoots) #2 (1 shoot)	#1 (3 shoots) #2 (2 shoots) (2 roots)	#2 (2 shoots) #3 (2 shoots)
KIN-0.02 IAA-2.0	#1 (1 root) #2 (4 roots) #3 (6 roots) #4 (23 roots)	#1 (2 roots) #5 (1 root)	#5 (4 roots)
KIN-1.0 IAA-1.0	No developments	#1 (3 shoots)	No developments
KIN-0.2 IAA-2.0	No developments	No developments	No developments
KIN-1.0 IAA-2.0	No developments	No developments	No developments

[1]Observations listed according to explant no. (explants numbered relative to original position within the scape - low numbered explants originally in the upper portion of the scape).

APPENDIX TABLE 8

PRODEDURE USED FOR THE INDUCTION OF FLOWERING OF

Hemerocallis cv. "Queen of May"

Phase 1: Growth Chamber

7 days

8 hour "day".....65°F

16 hour "night".....55°F

Phase 2: Growth Chamber

6 weeks

8 hour "day".....55°F

16 hour "night".....42°F

Phase 3: Greenhouse

4-7 weeks

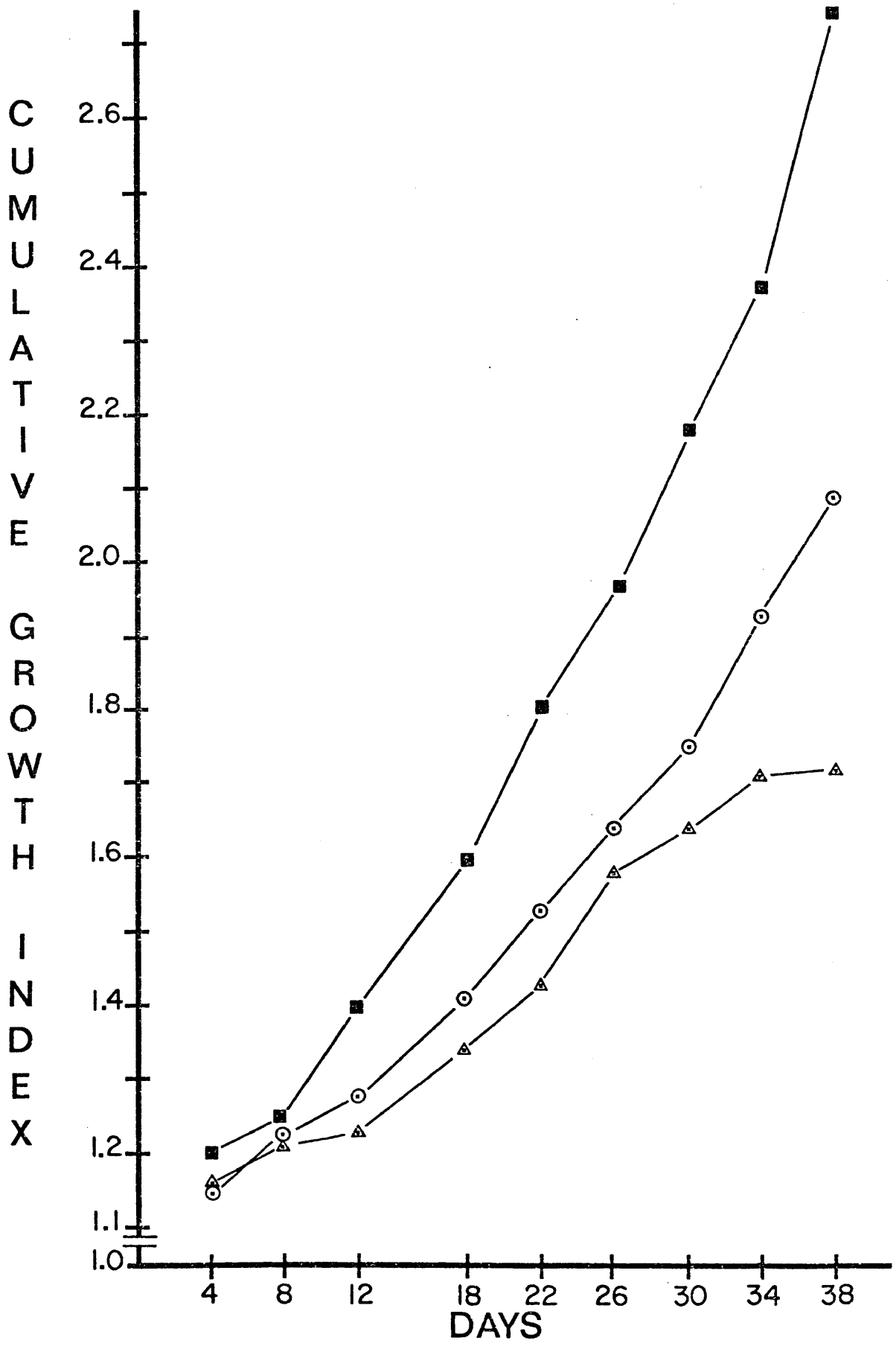
16 hour day- room temp. (approx. 70°F)

8 hour night- " " " "

APPENDIX FIGURE 1: Cumulative Growth Index (see Appendix Table 5) calculated from the average fresh weight increase (Appendix Table 4) of L. regale callus in response to standard and experimental concentrations of 2,4-D. Groups referred to by their respective experimental concentrations of 2,4-D (mg/l).

Legend:

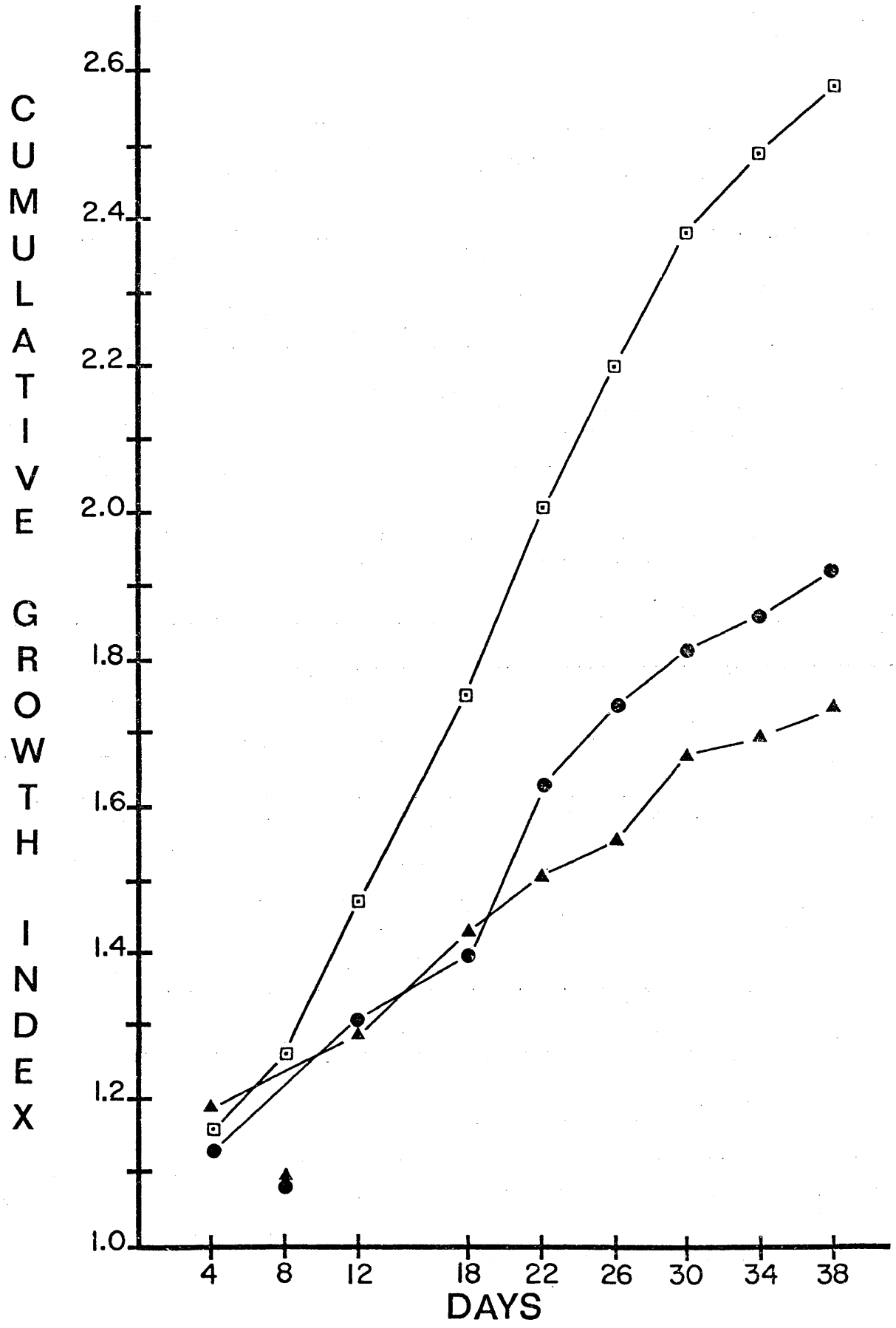
■ – 0.5 group
⊙ – 1.0 group (control)
▲ – 3.0 group


CUMULATIVE GROWTH INDEX
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.1
1.0
4
8
12
18
22
26
30
34
38
DAYS

APPENDIX FIGURE 2: Cumulative Growth Index (see Appendix Table 5) calculated from the average fresh weight increase (Appendix Table 4) of L. regale callus in response to standard and experimental concentrations of 2,4-D. Groups referred to by their respective experimental concentrations of 2,4-D (mg/l).

Legend:

⊡ – 5.0 group
● – 7.0 group
▲ –10.0 group


CUMULATIVE GROWTH INDEX
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
4
8
12
18
22
26
30
34
38
DAYS

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